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Malate- and Pyruvate-Dependent Fatty Acid Synthesis in Leucoplasts from Developing Castor Endosperm[1]

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ABSTRACT

Leucoplasts were isolated from the endosperm of developing castor (*Ricinis communis*) endosperm using a discontinuous Percoll gradient. The rate of fatty acid synthesis was highest when malate was the precursor, at 155 nanomoles acetyl-CoA equivalents per milligram protein per hour. Pyruvate and acetate also were precursors of fatty acid synthesis, but the rates were approximately 4.5 and 120 times less, respectively, than when malate was the precursor. When acetate was supplied to leucoplasts, exogenous ATP, NADH, and NADPH were required to obtain maximal rates of fatty acid synthesis. In contrast, the incorporation of malate and pyruvate into fatty acids did not require a supply of exogenous reductant. Further, the incorporation of radiolabel into fatty acids by leucoplasts supplied with radiolabeled malate, pyruvate, or acetate was reduced upon coincubation with cold pyruvate or malate. The data suggest that malate and pyruvate may be good *in vivo* sources of carbon for fatty acid synthesis and that, in these preparations, leucoplast fatty acid synthesis may be limited by activity at or downstream of the acetyl-CoA carboxylase reaction.

Storage lipids make up 45 to 50% of the dry weight of castor bean (*Ricinus communis*) endosperm (9, 15). During development, the conversion of sucrose to triacylglycerols is the major metabolic activity of this tissue (9). Dennis (11) presented a model indicating the possible pathways of triacylglycerol synthesis from sucrose. The model is primarily based on enzyme compartmentation studies (12, 17, 22, 23, 27) and suggests that glycolytic intermediates may cross the leucoplast membrane and serve as carbon skeletons for fatty acid synthesis. Acetate is another possible precursor.

Acetate incorporation into fatty acids has been used to study the cofactor requirements for fatty acid biosynthesis (6, 16, 18, 25, 26). It is unlikely that acetate is the *in vivo* carbon precursor of fatty acid synthesis because of its relatively low cellular concentration and the low efficiency of acetate utilization for *de novo* fatty acid synthesis in oil seeds (21). On the other hand, glycolytic intermediates are present in relatively large concentrations and the activities of the cytosolic or leucoplast glycolytic enzymes are more than adequate to account for the triacylglycerol synthesis of castor endosperm (17). However, glycolytic intermediates have not been well characterized as carbon sources for fatty acid synthesis (18). In this study, we present data regarding the kinetics and exogenous cofactor requirements for fatty acid synthesis in isolated leucoplast preparations using pyruvate, malate, and acetate as carbon sources. We show that malate and pyruvate support much higher rates of fatty acid synthesis than does acetate and the metabolism of both these substrates alleviates any requirement for externally added reductant. Based on the measurement of leucoplast-associated $NADP^+$-malic enzyme activity, we suggest that cytosolic malate may be a source of carbon for fatty acid synthesis *in vivo*.

MATERIALS AND METHODS

Plant Material

Castor plants (*Ricinus communis* L. cv Baker 296) were greenhouse grown under natural light supplemented with 16 h fluorescent light.

Leucoplast Isolation

Leucoplasts were isolated by a method similar to that reported by Boyle *et al.* (5). Endosperm of castor seeds, stages 5 to 6 (14), were dissected, placed in homogenization buffer (1:2, w/v), and ground with a mortar and pestle containing sea sand. The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 5 min. The supernatant ($S_{0.5}$) was centrifuged at 6000*g* for 10 min. The resulting pellet (P_6) was resuspended in 5 mL homogenization buffer and layered onto a discontinuous Percoll gradient. The gradient consisted of 5 mL of 35% PBF-Percoll, 10 mL of 22% PBF-Percoll, and 7.5 mL of 10% PBF-Percoll. Each layer contained 50 mM Hepes-KOH (pH 7.5), 0.4 M sorbitol, 0.4 mM EDTA, 1 mM $MgCl_2$ (5). The gradient was centrifuged in a swing-out rotor at 9200*g* for 8 min. The band of leucoplasts at the 22 to 35% Percoll interface was collected and used directly in the subsequent experiments. The direct use of this fraction reduced the damage to the leucoplasts as judged by the enolase intactness assay.

Determination of Leucoplast Intactness

A comparison between the enolase activity in leucoplasts resuspended in osmoticum in the presence or absence of Triton X-100 was used to determine the intactness of each

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preparation. Enolase activity was measured using an enzymatic-coupled assay and a dual wavelength spectrophotometer (Sigma ZFP22). The assay mixture contained 50 mM Tes (pH 7.5), 0.4 M sorbitol, 10 mM $MgCl_2$, 0.05 mM NADH, 3 mM ADP, 1 mM 2-phosphoglyceric acid, 2 units pyruvate kinase, and 2 units lactate dehydrogenase (final volume 600 μL) and the absorbance at 340 nm was followed. The proportion of "leaky" leucoplasts was taken as the ratio of the initial enolase activity to that following the disruption of the leucoplasts by adding 10 μL of 10% Triton X-100.

Marker Enzymes and Protein Assay

Marker enzyme activities and protein concentrations were determined for the $S_{0.5}$, P_6, and leucoplast fractions of each preparation. Acetyl-CoA carboxylase (EC 6.4.1.2), NAD-isocitrate dehydrogenase (EC 1.1.1.41), and catalase (EC 1.11.1.6) were used as marker enzymes for the leucoplast, mitochondrial, and peroxisomal fractions, respectively. The assay conditions for these assays are as reported elsewhere (4, 8). Briefly, acetyl-CoA carboxylase was assayed by a [^{14}C] HCO_3^- technique. Samples were incubated in buffer containing 50 mM Tris (pH 8.0), 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM DTT, 1 mM ATP, 1 mM acetyl-CoA, and 10 mM $NaHCO_3$ (specific activity, 0.5 mmol/mCi). The incubations lasted for up to 2.5 min and were stopped with ethanol/formic acid/water (80:5:20, v/v). The samples were dried down three times and the dpm of each sample determined by liquid scintillation counting. NAD-isocitrate dehydrogenase was assayed spectrophotometrically using a dual wavelength spectrophotometer (Sigma, ZFP22). The assay buffer contained 50 mM Tes (pH 7.5), 4 mM D,L-isocitrate, 2 mM $MnSO_4$, and 1 mM NAD. Catalase activity was assayed through the production of O_2 in a Clark-type O_2 electrode (Hansatech Ltd., King's Lynn, England). The reaction buffer contained 100 mM potassium phosphate (pH 7.5) and 0.3% (final concentration) H_2O_2.

Protein concentrations were measured according to Bradford (7) using a Bio-Rad assay kit and γ-globulin as a standard. To remove the BSA present in the homogenization buffer and Percoll, the fractions were washed three times in buffer containing 50 mM Hepes-KOH (pH 7.5), 1 M sorbitol, and 1 mM $MgCl_2$. The washed material was suspended in 10% TCA for 10 min, pelleted, and resuspended in 0.1 N NaOH.

Fatty Acid Synthesis

The rate of fatty acid synthesis by isolated leucoplasts was determined as described elsewhere (6, 18). Leucoplasts were incubated in media consisting of 50 mM Tes (pH 7.5), 0.4 M sorbitol, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 20 mM $KHCO_3$. Unless otherwise indicated, ATP, NADH, and NADPH were added to concentrations of 2, 0.1, and 0.1 mM, respectively. The radiolabeled carbon sources for these experiments were: [^{14}C]U-malate (Amersham, CFB.42); [^{14}C]2-pyruvate (New England Nuclear NEC 256); [^{14}C]6-glucose (New England Nuclear, NEC 045X); and [^{3}H]acetate (New England Nuclear NET 003). The radiolabeled carbon sources were diluted with cold substrate to the desired specific activity of 1 mmol/mCi for malate, pyruvate, and glucose and 50 μmol/mCi for acetate. Incubations (final volume, 200 μL) lasted for 60 min and were then stopped with 400 μL of 0.65 N KOH. The radiolabel incorporated into fatty acids was partitioned into $CHCl_3$ by adding 750 μL of $CHCl_3$/MeOH (2:1). In the case of ^{14}C-substrate, the $CHCl_3$ layer was washed three to five times with 750 μL of 0.5 M KCl, mixed with Aquasol II (New England Nuclear), counted using a liquid scintillation counter (LKB) and quench-corrected using an external standard. Samples containing ^{3}H were treated as above except that they were dried and resuspended in 250 μL of $CHCl_3$ before adding the scintillation cocktail. The $CHCl_3$ did not quench the counting of ^{3}H or ^{14}C samples (data not shown).

[^{14}C]CO_2 release when leucoplasts were supplied with uniformly labeled [^{14}C]malate was measured by incubating leucoplasts with 2.5 mM malate (specific activity, 1 mmol/mCi) for 1 h in the center well of a sealed flask. Incubations were stopped with 200 μL of 1.0 N HCl and left overnight. The [^{14}C]CO_2 released by the leucoplasts was trapped in 1 mL of β-phenylethylamine placed outside the center well of the incubation flask. The base was removed and mixed with 5 mL of Aquasol II and counted as above. The incubation mixture was alkalized with 200 μL of 2.5 N KOH and used to determine the incorporation of malate into fatty acids as described above.

Malic Enzyme Assay

The NAD- and NADP-dependent malic enzyme (EC 1.1.1.39 and EC 1.1.1.40, respectively) activity of the $S_{0.5}$, P_6, and leucoplast fractions were measured using a stopped assay. Samples (100 μL) were incubated in 50 mM Hepes-NaOH buffer (pH 7.0) containing 20 mM KCl, 6 mM $MnCl_2$, 5 mM L-malate, 0.75 mM EDTA, and 1 mM $NADP^+$ or NAD^+. Incubations lasted 30 min, a period of linear malic enzyme activity (data not shown), and the incubation stopped with the addition of 100 μL of 1 N HCl. The samples were neutralized with 5 N KOH/1 M triethanolamine. The pyruvate produced during the incubations was assayed using a standard enzymatic coupled technique and a dual wavelength spectrophotometer (Sigma ZFP22) (4). The activity of NADP-malic enzyme was also measured directly with a dual wavelength spectrophotometer. The assay mixture was the same as listed above. The assays were started with the addition of extract. Potential interference from malate dehydrogenase activity was controlled for by leaving out $MnCl_2$ from the initial reaction mixture (10). The activity of NADP-malic enzyme of "leaky organelles" in the leucoplast fraction was also determined. Similar to the enolase intactness assay, the initial rate of NADP-malic enzyme (due to "leaky" leucoplasts) was measured in the presence of 0.4 M sorbitol. The total activity of malic enzyme in the sample was determined after adding 10 μL of Triton X-100 to the reaction mixture.

Metabolite Analyses

The concentrations of malate and pyruvate in endosperm tissue was determined using standard techniques (4, 24). Endosperm tissue was frozen in liquid N_2, ground in a mortar and pestle, extracted in 10% $HClO_4$, and neutralized with 5 N KOH/1 M triethanolamine. The metabolites were measured using standard assays (4, 24).

RESULTS

Leucoplast Isolation

Discontinuous Percoll density gradients have been used to provide enriched leucoplast preparations from developing castor endosperm (5, 19). The technique has been shown to effectively separate leucoplasts from the cytosolic, mitochondrial, and peroxisomal fractions. The acetyl-CoA carboxylase activity and fatty acid synthetic rates (Figs. 1, 2) of the material from the 22 to 35% Percoll interface indicated that this fraction was enriched with leucoplasts. The specific activity of the Percoll fraction acetyl-CoA carboxylase (3.43 nmol·mg^{-1} organellar protein·min^{-1} or 205.8 nmol·mg^{-1} organellar protein·h^{-1}) doubled from that in the $S_{0.5}$ fraction. In contrast, the specific activities of NAD-isocitrate dehydrogen-

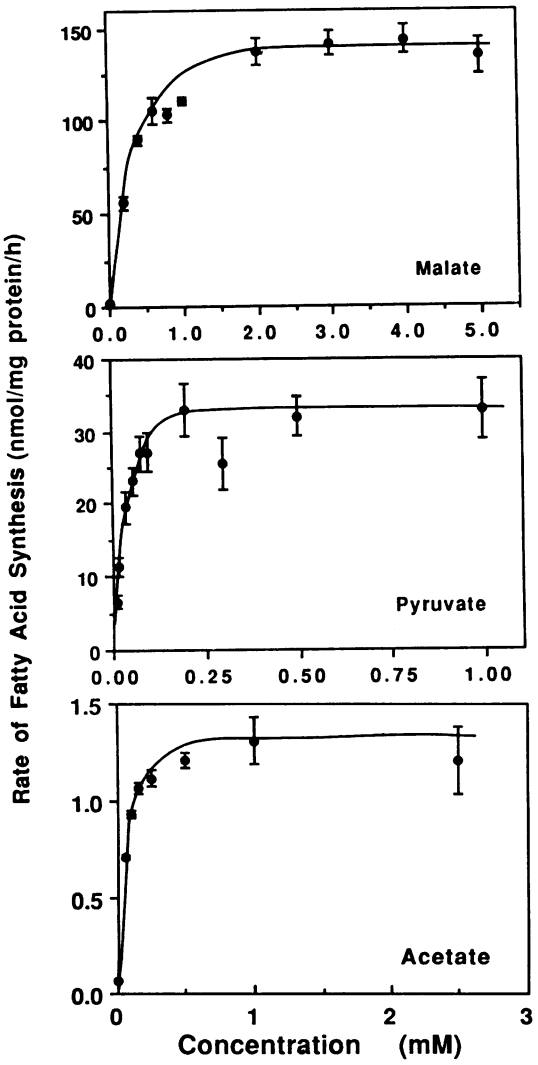

Figure 1. Rate of fatty acid synthesis *versus* substrate concentration of isolated leucoplasts from developing castor endosperm. The leucoplasts were supplied with 2 mM ATP, 0.1 mM NADH, 0.1 mM NADPH, and either [^{14}C]malate, [^{14}C]pyruvate, or [^{3}H]acetate. Symbols represent the means and standard deviations of five determinations.

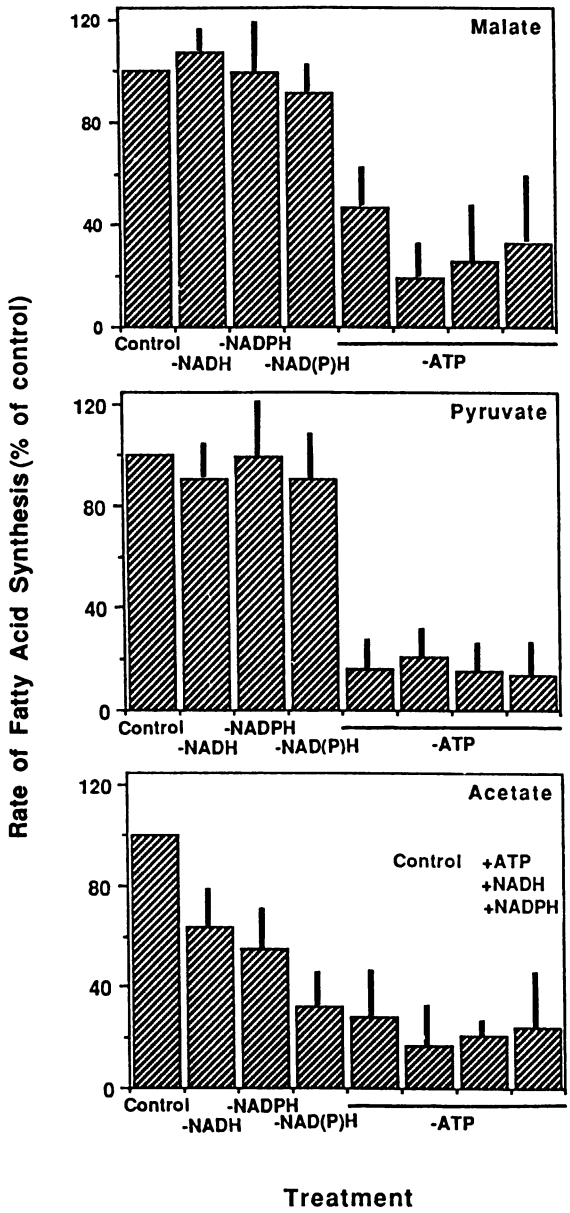

Figure 2. Cofactor requirements for fatty acid synthesis of castor endosperm leucoplasts supplied with [^{14}C]malate, [^{14}C]pyruvate, or [^{3}H]acetate as a carbon source. The control rate of fatty acid synthesis, in the presence of 2 mM ATP and 0.1 mM NADH and NADPH, was set at 100%. The −ATP treatments include: column 5, +NADH; column 6, +NADPH; column 7, +NADH and NADPH; column 8, carbon source alone. The bars represent the means and standard deviations of three to six separate determinations.

ase (17.05 nmol·mg^{-1} organellar protein·min^{-1}), a mitochondrial marker, and catalase (102 μmol·mg^{-1} organellar protein·min^{-1}), a peroxisomal marker, decrease four- and threefold, respectively. The leucoplasts were used directly from the Percoll gradient and were 75 to 80% intact as determined by the enolase assay. Similarly, 78 to 82% of the NADP-malic enzyme activity was found to be within the leucoplasts.

Fatty Acid Synthesis

Isolated leucoplasts from developing castor endosperm synthesize fatty acids from acetate, pyruvate, or malate (Fig. 1). The maximum rate of fatty acid synthesis from malate was 155 nmol acetyl-CoA equivalents·mg^{-1} protein·h^{-1}. This rate was up to 4.5 and 120 times greater than when leucoplasts were supplied with pyruvate or acetate, respectively (Fig. 1). Isolated leucoplasts supplied with [^{14}C]Glc did not synthesize any detectable labeled fatty acids (data not shown). The half saturation constant for malate incorporation into fatty acids was 338 μM, approximately 10 times that of pyruvate (34 μM) or acetate (35 μM) (Fig. 1).

Maximal rates of acetate incorporation into fatty acids by isolated leucoplasts required externally added ATP, NADH, and NADPH. The absence of any of these cofactors resulted in a marked decrease in the rate of fatty acid synthesis (Fig. 2). In contrast, the incorporation of malate and pyruvate into fatty acids did not require externally added reductant but did require ATP (Fig. 2).

The amount of radiolabel incorporated into fatty acids by leucoplasts supplied with [^{14}C]malate was reduced by up to 75% when 10 mM [^{12}C]pyruvate was added to the incubation mixture (Table I). Similarly, the amount of radiolabel incorporated into fatty acids by leucoplasts supplied with [^{14}C]pyruvate was reduced 40% upon coincubation with 10 mM [^{12}C]malate. In contrast, the amount of radiolabel incorporated into fatty acids by leucoplasts supplied with either [^{14}C]malate or [^{14}C]pyruvate was not reduced when coincubated with up to 10 mM acetate. The radiolabel entering the fatty acid pool of leucoplasts supplied with [^{3}H]acetate was reduced by about 25 and 60%, respectively, when coincubated with [^{12}C]malate or [^{12}C]pyruvate.

CO_2 Evolution from Malate

Over a 1 h incubation with 2.5 mM malate (specific activity, 1 mmol/mCi), 3.7 mol of malate-derived CO_2 was evolved for every mol of malate incorporated into fatty acids.

Malate and Pyruvate Concentrations

Castor endosperm tissue was found to contain approximately 5 mM malate and 50 μM pyruvate.

DISCUSSION

Fatty Acid Synthesis

Leucoplasts isolated from developing *R. communis* endosperm can synthesize fatty acids when supplied with malate, pyruvate, or acetate as a carbon source (Fig. 1). The rate of fatty acid synthesis from acetate was about 1.3 nmol·mg^{-1} protein·h^{-1}. This is lower than the rate of acetate incorporation into fatty acids of leucoplasts isolated by differential centrifugation or sucrose gradient centrifugation (18) but comparable to the rate of 2.1 nmol·mg^{-1} protein·h^{-1} calculated from the data presented by Boyle *et al.* (6), who used the same tissue and techniques. The rates of fatty acid synthesis by leucoplasts supplied with malate or pyruvate were about 120- and 25-fold higher than when supplied with acetate (Fig. 1). Miernyk and Dennis (18) and Yamada and Usami (26) also found that pyruvate supported higher rates of fatty acid synthesis than did acetate.

The half saturation constants of malate and pyruvate incorporation into fatty acids were about 340 and 35 μM, respectively (Fig. 1). The concentration of malate within the developing endosperm was about 5 mM and that of pyruvate was 50 μM. Although the compartmentation of these substrates is unknown, it seems likely that both malate and pyruvate are present in the cytosol at high enough concentrations to support high rates of fatty acid synthesis *in vivo*.

The incorporation of acetate into fatty acids requires exogenous NADH, NADPH, and ATP. The requirement for exogenous reducing power was alleviated when either malate or pyruvate were used as the source of carbon for fatty acid synthesis (Fig. 2), suggesting that these substrates also provide all of the necessary reducing power for fatty acid synthesis. The incorporation of one acetyl-CoA unit into a fatty acid chain requires two molecules of NAD(P)H (2, 11). It has been

Table I. *Percentage of Radiolabel Incorporated into Fatty Acids of Leucoplasts Incubated with ^{14}C-labeled Malate or Pyruvate, or [^{3}H] Acetate, and Coincubated with Varying Concentrations of Unlabeled Malate, Pyruvate, or Acetate*

Incubations with radiolabeled malate or pyruvate contained 2 mM ATP, and those with radiolabeled acetate contained 2 mM ATP and 0.1 mM NADH and NADPH. The data are the means and standard deviations of 10 measurements.

Labeled Substrate	Unlabeled Substrate	Label in Fatty Acids
		% of control
Malate (2 mM)		100 ± 7
	Pyruvate (35 μM)	112 ± 8
	Pyruvate (1 mM)	43 ± 7
	Pyruvate (10 mM)	25 ± 6
	Acetate (35 μM)	103 ± 16
	Acetate (1 mM)	113 ± 9
	Acetate (10 mM)	111 ± 6
Pyruvate (0.5 mM)		100 ± 17
	Malate (0.35 mM)	75 ± 24
	Malate (3 mM)	73 ± 14
	Malate (10 mM)	60 ± 6
	Acetate (35 μM)	119 ± 15
	Acetate (1 mM)	111 ± 13
	Acetate (10 mM)	83 ± 8
Acetate (1 mM)		100 ± 19
	Malate (0.35 mM)	62 ± 11
	Malate (3 mM)	78 ± 18
	Malate (10 mM)	76 ± 16
	Pyruvate (35 μM)	66 ± 10
	Pyruvate (1 mM)	60 ± 10
	Pyruvate (10 mM)	42 ± 13

suggested that there is a requirement for both NADPH and NADH (13). The conversion of pyruvate to acetyl-CoA, via leucoplast pyruvate dehydrogenase complex, will supply one molecule of NADH. In the case of malate, NADPH could be supplied through the conversion of malate to pyruvate by NADP-linked malic enzyme. In this study, we found that the specific activity of NADP-linked malic enzyme increased from the P_6 to the leucoplast fraction. Further, the proportion (78–82%) of NADP-malic enzyme activity within the leucoplasts from the Percoll fraction was the same as enolase (75–80%), suggesting that leucoplasts from developing castor endosperm possess an NADP-linked malic enzyme. It has been suggested that the NADP-linked form of malic enzyme is predominantly a plastidic enzyme (T. ap Rees, personal communication). The specific activity of the NADP-malic enzyme from the leucoplast fraction was 8.25 $\mu mol \cdot mg^{-1}$ protein $\cdot h^{-1}$, which is greatly in excess of that required to support the malate-dependent rate of fatty acid synthesis.

This study had shown that malate, of all the substrates tested, supported the highest rates of fatty acid synthesis and that its metabolism is capable of providing all the reductant required for carbon incorporation into fatty acids. It is well established that leucoplasts from castor endosperm contain both the glycolytic (11) and oxidative pentose phosphate pathways (1, 11, 13). The identification of exogenous malate as an important substrate for fatty acid synthesis illustrates that there may be sources of carbon for fatty acid synthesis in addition to those provided directly from glycolytic intermediates. Although malate is capable of providing the carbon and reducing power for fatty acid synthesis, there is still a requirement for ATP, and it is possible that glycolysis within the leucoplasts provides this cofactor (6).

Although the rates of fatty acid synthesis from malate or pyruvate were much greater than those reported by acetate, these observations do not necessarily discount acetate as being an effective *in vivo* source of carbon for fatty acid synthesis. One explanation for the low rates of acetate-dependent fatty acid synthesis is that exogenously supplied reductant may not be entering the leucoplast at rates sufficient to meet the demands for fatty acid synthesis. It has been shown that isolated spinach chloroplasts will only assimilate acetate into fatty acids when ATP and NAD(P)H are provided endogenously through the photosynthetic electron transport chain (20). The endogenous production of NAD(P)H associated with malate and pyruvate metabolism may, in part, account for the high rates of fatty acid synthesis supported by these substrates.

Model of Malate and Pyruvate Flow to Fatty Acids

Based on our results, we propose that one pathway of carbon flow to fatty acid synthesis may be as outlined in Figure 3. The model indicates that the sucrose imported into the endosperm is metabolized to PEP[4] in the cytosol. PEP can be metabolized further in two ways. First, pyruvate kinase may catalyze the conversion of PEP to pyruvate, which then crosses the leucoplast membrane. Second, PEP may be carboxylated via PEP carboxylase to oxaloacetate and, with the

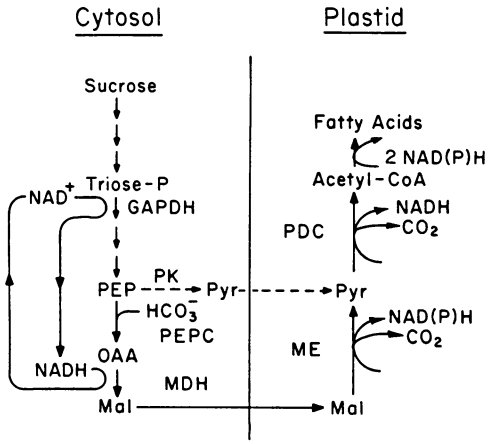

Figure 3. Proposed model of malate and pyruvate utilization during fatty acid synthesis in developing castor endosperm. The model shows the production, requirements, and shuttling of reductant in the endosperm tissue.

action of cytosolic malate dehydrogenase, converted to malate, which then crosses the leucoplast membrane. The model indicates that pyruvate supplies one reducing equivalent within the leucoplast by action of the pyruvate dehydrogenase complex. Malate, on the other hand, supplies two reducing equivalents through the sequential action of NADP-linked malic enzyme and the pyruvate dehydrogenase complex. Several predictions can be made from the model.

The model indicates that malate-dependent radiolabel incorporation into fatty acids should decrease upon coincubation with unlabeled pyruvate. Similarly, pyruvate-dependent radiolabel incorporation into fatty acids should decrease upon coincubation with unlabeled malate. This is what was found. The radiolabel incorporated into fatty acids was reduced by up to 75% when [^{12}C]pyruvate was added to [^{14}C]malate incubations, and reduced by up to 40% when [^{12}C]malate was added to [^{14}C]pyruvate incubations (Table I). Further, the addition of unlabeled malate or pyruvate to leucoplasts incubated with [^{3}H]acetate also results in a decrease in the radiolabel entering into the fatty acid pool (Table I). These data indicate that, in these preparations, fatty acid synthesis is limited at, or downstream of, the acetyl-CoA carboxylase reaction. The addition of unlabeled acetate to ^{14}C-labeled malate or pyruvate incubations did not have an effect on the radiolabel entering into the fatty acid pool (Table I). This is not inconsistent with the above conclusion because the rate of acetate incorporation into fatty acids is extremely low compared with that of malate or pyruvate (Fig. 1). Interestingly, the presence of unlabeled malate or pyruvate did not completely inhibit the incorporation of radiolabeled acetate into the fatty acid pool (Table I). Perhaps the metabolism of malate or pyruvate within leucoplasts provide sufficient reducing power to allow for the incorporation of acetate into the fatty acid pool.

If malate is an *in vivo* carbon source for fatty acid synthesis, each molecule of uniformly labeled malate incorporated into fatty acid should release two molecules of [^{14}C]CO_2 through the action of NADP-malic enzyme and the pyruvate dehydro-

[4] Abbreviation: PEP, phospho*enol*pyruvate.

genase complex. When $^{14}CO_2$ release was measured, it was found that 3.7 CO_2 were produced per acetyl-CoA equivalent incorporated into fatty acid. This suggests either that there is a build up of intermediates of fatty acid synthesis that are not extracted by chloroform or that pyruvate and malate are also being respired to CO_2.

The model also predicts that developing castor endosperm should exhibit high rates of PEP carboxylase activity, which would produce malate for fatty acid synthesis. It has been found that crude extracts from developing endosperm have very high activities of PEP carboxylase (2.7 $\mu mol \cdot g^{-1} \cdot min^{-1}$, a value about three times greater than that for NADP-malic enzyme; 22a) and also exhibit high rates of dark carbon fixation (3).

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